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Combined Exposure to Ozone and Nitrogen Dioxide

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A study of rats acutely exposed to ozone (0.5–2.0 ppm) or nitrogen dioxide (2–20 ppm) for 2 hr and sacrificed immediately thereafter shows little similarity in the individual biochemical effects of these pollutants. No evidence of nitrogen dioxide-induced lipid peroxidation was observed. Of interest is the finding that inhalation of nitrogen dioxide increases the extent to which concanavalin A agglutinates alveolar macrophages while ozone has exactly the opposite effect.

Studies in the literature have suggested that the gaseous pollutants ozone and nitrogen dioxide have many similarities in their effects. In addition to being lung irritants producing death in pulmonary edema at high concentrations, the similarities include the fact that they are both relatively insoluble and therefore penetrate deeply into the respiratory tract. They have an almost identical effect of potentiating bacterial infections at low concentrations, in both cases apparently due to an interference in lung bacteriocidal capabilities (*1–4*). Both are oxidants and are reported to produce lipid peroxidation *in vitro* and in the lungs of animals inhaling relatively low concentrations of either (*5–9*). Long-term exposure to either results in pathological changes suggestive of chronic respiratory disease (*10, 11*). Furthermore, there is cross tolerance in that sublethal exposure to either ozone or nitrogen dioxide protects against subsequent exposure to lethal levels of both (*12, 13*).

Although it is clear that differences also exist between ozone and nitrogen dioxide, it was believed at the inception of this part of the U.S.-U.S.S.R. cooperative environmental health program that study of these two pollutants would be a useful first approach to the problem of assessing methodology for evaluating the combined effects of environmental pollutants. This supposition was predicated on there being similar observable biochemical effects of ozone and nitrogen dioxide exposure for which dose response curves could be constructed for the individual pollutants alone. This would serve as a basis for observations of effects following combined exposure to various levels of ozone and nitrogen dioxide. The findings would then be utilized to assess the appropriate methodology for predicting combined effects of two similar pollutants.

The first series of studies in this laboratory evaluating the combined effect of ozone and nitrogen dioxide utilized an *in vitro* model system in which human red blood cells were exposed to ozone and nitrogen dioxide, alone and in combination (*14*). For certain parameters, generally additive effects were observed. However, for other parameters the effects varied from protective to synergistic, depending upon the pollutant concentration, duration, and sequence of exposure. This led to the preliminary conclusion that it might be misleading to estimate the combined effects of ambient levels of two pollutants by extrapolation from a study in which the effects of a single dose of each was compared with those occurring after combined exposure to the same doses of both.

It must be emphasized that the red cell model system was suitable for this phase of the assessment of methodology for determining the combined effects of pollutants in that parameters were available which were altered in the same direction, although to differing extents, by both ozone and nitrogen dioxide. In order to perform similar studies in an *in vivo* system in which animals inhale both ozone and nitrogen dioxide, it is also necessary to have measurable parameters which are affected by both of these pollutants alone. However, in our studies

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to date we have thus far been unable to identify a biochemical parameter which is affected by both ozone and nitrogen dioxide following acute exposure to either pollutant and which would therefore be suitable for assessing combined effects. In fact, for one of the parameters chosen, the agglutination of alveolar macrophages by concanavalin A (Con A), ozone and nitrogen dioxide appear to have opposite effects.

The studies were performed in male Sprague-Dawley rats (200-250 g) who were acutely exposed to either ozone (0.5–2.0 ppm) or nitrogen dioxide (2–20 ppm) for 2 hr. Immediately following exposure the animals were sacrificed under ether anesthesia by exsanguination and the lung and trachea dissected free. Endobronchial lavage was performed by the procedure of Brain and Frank (*15*) using divalent cation-free phosphate buffered (0.01*M*) isotonic saline, pH 7.4. The lungs were then homogenized and strained through gauze to prepare a whole lung homogenate (WLH). An aliquot of the WLH was saved, and the remainder was centrifuged at 9000 *g* for 20 min in 0.25*M* sucrose–0.01*M* Tris–0.02*M* EDTA, pH 7.4. The supernatant was then centrifuged at 109,000 *g* for 1 hr and the pellet resuspended in 0.01*M* Tris–0.02M EDTA, pH 7.4, before recentrifugation at this speed and resuspension in Tris–EDTA. This fraction, designated the fluffy layer, is greatly enriched in plasma membranes, as shown by a 10-12 fold increase in 5′-nucleotidase activity.

The endobronchial lavage sample was centrifuged at 3000 *g* for 10 min and the supernatant removed for study of the cell-free bronchial wash sample. The pellet was resuspended in PBS and washed twice more in this buffer. Before study of Con A agglutination, the alveolar macrophage sample was purified by centrifugation in a hypaque-ficoll gradient (*16*). This was necessary to remove red cells and granulocytes which were present in far larger amounts in samples from pollutant exposed rats. The resultant preparation was still contaminated with lymphocytes (< 10%), but this was similar in all groups. Alveolar macrophage agglutination was measured following incubation with 0, 100 and 500 μg/ml Con A for 30 min by counting agglutinated and nonagglutinated cells in a hemocytometer. Lipid peroxidation was assayed by the thiobarbituric acid test (*17*), protein and nonprotein sulfhydryl groups by a minor variation of the procedure of Sedlak and Lindsay (*18*), and tryptophan content and native protein fluorescence as previously described (*19*).

Exposure to 0.5, 1.0, or 2.0 ppm ozone resulted in the production of lipid peroxides as would be expected from previous studies. However, only slight decreases in protein or nonprotein sulfhydryl levels were observed in various lung fractions, being somewhat more prominent in the fluffy layer. However, these differences in sulfhydryl levels are not statistically significant although continued study is in progress. This differs from the findings of other investigators (*20, 21*) but is not inconsistent with those reported by Menzel et al. (*22*). Evaluation of animals exposed to NO_2 concentrations up to and including lethal levels showed no evidence of lipid peroxidation, as measured by the thiobarbituric acid test. The observation of lipid peroxidation in NO_2 exposed animals by Thomas et al. (*9*) was made in animals sacrificed 16 hr after exposure rather than at the cessation of exposure. This might possibly explain the different observations. No effect of NO_2 exposure on lung protein or nonprotein sulfhydryl levels were observed. Native protein fluorescence in relation to cell membrane tryptophan levels was found not to be a useful parameter for study of combined effects of ozone and nitrogen dioxide in that nitrite, and nitro compounds in general, are nonspecific quenchers of native protein fluorescence.

In studies evaluating the agglutination of alveolar macrophages by Con A, ozone and nitrogen dioxide were found to give opposite responses. Alveolar macrophages obtained from rats inhaling 0.5–2.0 ppm ozone for 2 hr were less agglutinable by Con A, while alveolar macrophages obtained from rats inhaling 10–20 ppm NO_2 for 2 hr were more agglutinable (see Table 1). The latter observation may be pertinent to a recent report in which human alveolar macrophages obtained from cigarette smokers were more agglutinable than those obtained from nonsmokers (*23*). For both ozone and nitrogen dioxide the observed alteration in Con A agglutination was confirmed by *in vitro* studies, and agglutination by Con A was completely inhibited by α-methyl-D-mannopyranoside indicating the specificity of the effect. Preliminary findings show no significant change in the binding of radioactive Con A to alveolar macrophages following either ozone or nitrogen dioxide suggesting that the observed ef-

Table 1. Agglutination of rat alveolar macrophages by Concanavalin A following inhalation of 2 ppm ozone or 20 ppm nitrogen dioxide for 2 hr.[a]

Pollutant	Agglutination, % of controls[b]
Ozone	61 (52–71)
Nitrogen dioxide	170 (159–181)

[a] Concanavalin A, 500 μg/ml.

[b] Data, given as percent of the agglutination of nonexposed controls, are means of two exposure runs using two rats each. Ranges are in parentheses.

fects are due to alterations in the responsiveness of the alveolar macrophage cell membrane to Con A.

The original impetus for this study were reports suggesting that cell membrane fluidity was an important determinant of response to Con A. Inasmuch as low concentrations of both ozone and nitrogen dioxide appear to interfere with the ability of alveolar macrophages to kill bacteria and membrane fluidity appears to be a significant factor in macrophage function, it was hypothesized that observation of the response to Con A of alveolar macrophages from pollutant exposed animals might be a sensitive indirect means of determining a component of the bacteriocidal activity of alveolar macrophages. Whatever is the relationship of Con A agglutination to alveolar macrophage function, the present study clearly indicates that ozone and nitrogen dioxide have opposite effects. This parameter is therefore not suitable for this phase of studies of the combined effects of environmental pollutants.

It must be emphasized that the findings in the present study do not disprove the hypothesis that ozone and nitrogen dioxide have similar biochemical mechanisms of toxicity. The present data are pertinent only to 2-hr exposure periods in which the animals are immediately sacrificed. Further studies will evaluate different time periods of exposure as well as different sacrifice schedules. In addition, the interaction of ozone and nitrogen dioxide on observational parameters, such as lung weight and amount of edema, will also be assessed.

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